



AD

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C07H 21/04, C12Q 1/68 // C07K 15/04		A1	(11) International Publication Number: WO 91/09049 (43) International Publication Date: 27 June 1991 (27.06.91)
<p>(21) International Application Number: PCT/GB90/01979</p> <p>(22) International Filing Date: 19 December 1990 (19.12.90)</p> <p>(30) Priority data: 8928625.6 19 December 1989 (19.12.89) GB</p> <p>(71) Applicant (<i>for all designated States except US</i>): 3i RESEARCH EXPLOITATION LIMITED [GB/GB]; The Gate House, 2 Park Street, Windsor, Berkshire SL4 1LU (GB).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (<i>for US only</i>) : TABAQCHALI, Soad [IQ/GB]; 9 Kent Terrace, London NW1 4RP (GB). CLAYTON, Christopher, Leeson [GB/GB]; 11 School Lane, Aston, Stevenage, Herts SG2 7HA (GB). WREN, Brendan, William [GB/GB]; 5 Leaside Walk, East Side, Ware, Herts SG12 9AB (GB). KLEANTHOUS, Harold [GB/GB]; 40 Manor Court, York Way, Whetstone, London N20 0DR (GB).</p>		<p>(74) Agents: LAMBERT, Hugh, Richmond et al.; D. Young & Co., 10 Staple Inn, London WC1V 7RD (GB).</p> <p>(81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.</p> <p>Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	
<p>(54) Title: H. PYLORI SPECIFIC OLIGONUCLEOTIDES</p> <p>(57) Abstract</p> <p>Oligonucleotide sequences are disclosed specific to <i>H. pylori</i> urease and useful as DNA probes and primers in the detection of <i>H. pylori</i> infection in humans.</p>			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	ML	Mali
AU	Australia	FR	France	MN	Mongolia
BB	Barbados	GA	Gabon	MR	Mauritania
BE	Belgium	GB	United Kingdom	MW	Malawi
BF	Burkina Faso	GN	Guinea	NL	Netherlands
BG	Bulgaria	GR	Greece	NO	Norway
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	SD	Sudan
CF	Central African Republic	KP	Democratic People's Republic of Korea	SE	Sweden
CG	Congo	KR	Republic of Korea	SN	Senegal
CH	Switzerland	LI	Liechtenstein	SU	Soviet Union
CI	Côte d'Ivoire	LK	Sri Lanka	TD	Chad
CM	Cameroon	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark		Madagascar		
ES	Spain				

- 1 -

H. PYLORI SPECIFIC OLIGONUCLEOTIDES

This invention relates to oligonucleotides showing a substantially specific binding affinity towards genomic DNA from bacteria of the species Helicobacter pylori, until recently more usually known as Campylobacter pylori, and which are potentially useful as H. pylori DNA probes and primers for the PCR amplification and detection of H. pylori genomic DNA and RNA.

10 H. pylori (previously C. pylori), a gram-negative spiral microaerophilic bacteria, was first cultured from the human gastric mucosa in 1983 and has since been strongly implicated in the pathogenesis of gastritis and duodenal and peptic ulceration in man. Further evidence that C. pylori is responsible for gastritis is 15 provided by studies showing that gastritis improves after patients receive therapy directed against C. pylori infection, and the fact that both the frequency of gastritis and C. pylori infection increase with age. Patients colonised with C. pylori have been found to elicit a specific antibody response and the frequency of detection of C. pylori 20 antibodies has also been found to increase with age.

The histopathology of gastritis associated with C. pylori is well characterised. The organism is closely associated with the mucosa in the gastric antrum and is located beneath the mucus layer. C. pylori 25 appear to adhere to mucus-secreting epithelial cells, and adhesion "pedestals" have been revealed by electron microscopy analysis. Colonised gastric epithelium shows changes in appearance and there is partial or complete loss of microvilli.

30 Virulence determinants of C. pylori have not so far been identified, although a number of determinants possessed by this organism have been proposed as possible pathogenic factors. For example, high mobility by virtue of multiple flagella allow C. pylori to move rapidly by a corkscrew-like motion through highly viscous 35 fluids such as the mucus layer of the gut which normally poses a barrier to bacteria en route to the gut epithelium. Also, in the habitat of the stomach mucus, the ability of C. pylori to produce

- 2 -

extracellular urease allows the organism to metabolise urea and create an alkaline environment which enables its survival in an otherwise hostile environment, C. pylori normally being sensitive to low pH. C. pylori urease has been found to be toxic to tissue culture cells in vitro due to the production of ammonia, and it is possible that the reported extracellular cytotoxin produced by C. pylori may contribute to the histopathological appearance of the gut mucosa associated with C. pylori colonisation. Moreover, although patients colonised with C. pylori do produce an immune response to the organism, it appears that the organism is able to evade the host defences and cause persistent infection.

For these and other reasons, it would be highly desirable to have a reliable means of detecting C. pylori in clinical samples from a patient, for example gastric mucosa, saliva or faecal samples, as a means of early diagnosis of gastritis and peptic ulceration. This is provided in accordance with the present invention by means of oligonucleotides specific to C. pylori - and useful as probes and primers, for the detection of C. pylori.

Campylobacter probes have previously been disclosed in WO 86/04422 and EP-A-0 232 085 but not specifically for C. pylori. In WO 86/04422 DNA probes are disclosed derived from the chromosomal sequences of C. jejuni and C. coli and which, respectively, are capable of hybridising with at least 80% of bacteria from the species C. jejuni and C. coli, preferably at least 90%, with no ability to hybridise with bacteria not in the genus Campylobacter. This would seem to suggest some ability to hybridise with Campylobacter other than C. jejuni and C. coli, but few examples are given and there is no suggestion of any hybridisation ability at all towards C. pylori, but in any case such probes would seem somewhat lacking in species specificity and, for that reason, of little value as a diagnostic tool.

EP-A-0 232 085 discloses DNA probes capable of hybridising to the rRNA of a variety of Campylobacter species, but not including C. pylori. The species mentioned are C. jejuni, C. coli, C. fetus, C. laridis, C. fetus subsp. venerialis and C. hyoilestinalis. The

- 3 -

preferred probes are complementary to the RNA of the 5S, 16S or 23S rRNA of Campylobacter, and especially to a sequence of 15 or more base pairs of the 16S rRNA sequence of C. jejuni, the base pair sequence of which is set out in the specification. Again, C. pylori probes are not 5 disclosed, and the probes that are disclosed would seem to lack the specificity essential to diagnostic work.

Campylobacter probes capable of specifically hybridizing to rRNA of C. jejuni, C. coli and C. laridis are also disclosed in EP-A-0350205 10 published after the present priority date. Whilst the majority of probes disclosed in that application show a substantial measure of specificity for the sub-group of Campylobacter species consisting of the group: C. jejuni, C. coli and C. laridis, at least one probe is disclosed which is far less specific and, in fact, shows a wide 15 hybridisation ability with rRNA from other microorganisms not only of the genus Campylobacter, but also of other genera, and included within that listing is the ability to hybridise with rRNA from microorganisms of the species C. pylori, which is the species of interest herein. That probe, probe No. 1105, is, however, hardly specific to the 20 selected group of C. jejuni, C. coli, and C. laridis, let alone C. pylori.

EP-A-0 350 392 falls in the same category as EP-A-0 350 205 above. In this case oligonucleotide probes are disclosed having 25 specificity towards DNA and RNA from microorganisms of the species C. coli, C. jejuni, C. fetus, C. laridis and C. upsaliensis. C. pylori probes are not disclosed.

Finally in the prior art, brief mention should also be made of 30 the serological detection and diagnosis of C. pylori infection by serological immunoassay and detection of C. pylori antigens and antigenic fragments. In this category are the disclosures of published international applications WO 89/08843 and WO 89/09407, and also EP-A-0 329 570.

35

There still exists in the art, the above prior art notwithstanding, a substantial need for a quick, efficient and reliable method

- 4 -

for the detection of C. pylori infection in animals, and particularly in humans, and the present invention seeks to fulfil that need, and is based on the identification and decoding of C. pylori urease gene fragment, the identification and isolation of which the present inventors reported in a paper entitled "Molecular Cloning and Expression of Campylobacter pylori Species Specific Antigens in Escherichia coli K-12" published in Infection and Immunity, 57, No. 3, 623-629 (1989). In this paper the present inventors disclosed the construction of a gene bank of C. pylori DNA in E. coli and the identification in subsequent screening, cloning and sub-cloning procedures, of a 2.7 kb TaqI DNA fragment encoding for the 66- and 31-kDA C. pylori antigens. Subsequent work has shown these cloned antigens to be substantially identical with the 66- and 31-kDA antigens forming part of the C. pylori urease enzyme, and not only that; but also that those antigens have been shown to be present in all C. pylori strains so far tested. Thus the possibility now arises in accordance with the present invention of constructing C. pylori DNA probes substantially specific to all strains of C. pylori, and potentially of great value in the early detection of C. pylori populations in the gastrointestinal tract and the early diagnosis of gastritis and possible ulceration, and their possible treatment with antibiotics, rather than long-term anti-secretory medication.

In accordance with the present invention, and following from the previous work, the 2.7 kb TaqI DNA fragment of C. pylori, referred to from hereon in its revised classification as H. pylori, encoding the 66-kDA and 31-kDA H. pylori antigens, the A and B sub-units of H. pylori urease, has been sequenced, giving rise to the possibility of constructing selected oligonucleotide sequences specific to H. pylori.

The complete sequence of the 2.7 kb TagI DNA fragment and the deduced amino acid sequence are set out in an appendix to and forming part of the present specification. The sequence corresponds substantially to the sequence disclosed in International Publication No. WO 90/04030 published after the present priority date.

35

That sequence shows two large open reading frames, codons 1 to 717 and 721 to 2400, encoding, respectively, proteins of calculated

- 5 -

molecular weights 26.657 kDa (sub-unit A) and 60.473 (sub-unit B).

Within that sequence we have now identified certain regions of the gene containing the most highly conserved sequences. These are the 5 regions nt. nos 255-714, 720-1020 and 1950 to 2397 inclusive, and especially the regions from about nt 286 to 714 and about nt 2070 to 2397 inclusive.

Based on that finding oligonucleotide probes and primers are now 10 provided showing substantially specific binding affinity towards the H. pylori gene encoding the A and B sub-units of the H. pylori urease gene, such oligonucleotides having a chain length of from 15 to 50 nucleotides, preferably 15-30, most preferably 15-25, and comprising a 15 sequence of at least 15 nucleotides which is the same as or complementary to a sequence of 15 or more nucleotides selected from any one of the above mentioned numbered nt sequences.

Tests have shown that oligonucleotides derived from these regions 20 of the gene are substantially specific to genomic DNA of all strains of H. pylori so far tested, with little or no cross-affinity to other urease positive enterobacter species. Such oligonucleotides are therefore highly preferred in the detection and identification of H. pylori infection in humans, for example, by means of saliva tests.

Especially preferred are oligonucleotides having a chain length 25 of from 15 - 30 nucleotides especially 15 - 24 and containing a sequence of at least 15 nucleotides corresponding or complementary to a sequence of at least 15 nucleotides commencing at about nt 298 or terminating at nt 714 and reproducing by PCR a fragment of 411 bp or 30 thereabouts, or commencing at about nt. 2074 or terminating at nt. 2397 and reproducing by PCR a fragment of about 323 bp. In particular pairs 35 of oligonucleotides of 15 to 30 nucleotides in length with one member of the pair having a sequence the same as or complementary to the sequence starting at about nt. 298 or 304 and the other the same as or complementary to the sequence terminating at nt. 714 have been found to amplify a 411 to 417 bp fragment that is common to all H. pylori strains so far tested, that does not occur in other ureases, and can

- 6 -

therefore be regarded as a specific marker for H. pylori. Similar results are obtainable with primer pairs amplifying a 323 bp fragment, and a characteristic 110 bp fragment.

5 As already indicated the oligonucleotides of the present invention will generally have a chain length (excluding any added tail, see later) of from 15 to 50 nucleotides. Various specific sequences are given hereinafter although it is to be understood that these are given merely by way of exemplification. The final selection of a
10 particular probe, or pair of probes will depend upon a number of factors, well understood in the art, and including amongst others the stringency requirements, i.e. the ability or otherwise of the probe to tolerate local mismatching with the complementary sequence in the target DNA. Obviously the longer the probe the better the ability to
15 withstand local mismatch without adversely affecting the hybridisation of the probe to the target DNA. However, the length of the probe always has to be balanced against other factors such as ease of synthesis. The factors affecting that choice are, however, well recognised and well within the capabilities of the person skilled in
20 the art.

Also, as the person skilled in the art will recognise, references herein to particular oligonucleotide probes and sequences in single stranded form, and which are written, as is required, reading from left to right i.e. from the 5' terminus to the 3' terminus, automatically include the complementary sequence. Not only that, but oligonucleotide sequences given herein as DNA sequences can equally well be constructed as RNA sequences with uracil (U) replacing thymine (T).

30 Whilst, as indicated, the generality of the present invention extends to DNA (and RNA) probes complementary to any sequence of nucleotide bases to be found in the highly conserved regions of the 2.7kb sequence set out hereinafter, certain sequences and pairs of sequences can be identified as being particularly preferred. For example, the pair of sequences:
35

- 7 -

(HPU1) 5'-GCCAATGGTA AATTAGTT-3' (nt. nos 304 to 321)

and

(HPU2) 5'-CTCCTTAATT GTTTTAC-3' (complementary to nt. nos 697 to 714).

5 Using this pair of primers a 411 base pair product has been amplified from urease gene A (nt 304-714) using a 26 cycle PCR, which allowed visualisation of the amplified product within 5 hours. Supernatants of 40 boiled *H. pylori* strains so far examined have given the 411bp amplified product on agarose gel electrophoresis.

10 Helicobacter mustelae and other urease positive bacteria have been found to be PCR negative. PCR detected as few as 100 *H. pylori* cells even in mixed cultures. Further PCR of initial amplified samples has been found to increase sensitivity 10-fold. A similar increase in sensitivity has been found by Southern hybridisation to an

15 oligonucleotide probe (HPUIS) derived from a sequence internal to the amplified product (5'-ATTGACATTG CGGGTAAC-3' nt. nos 559 to 576).

Other typical oligonucleotide sequences useful in accordance with the present invention are as follows:

20

25 units

(038) 5' CTCCACTACG CTGGAGAATT AGCTA 3'

24 units

25 HPU1¹ 5' ATTGAGGCCA ATGGTAAATT AGTT 3' (nt. nos 298 to 321)
HPU2¹ 5' CTCCTTAATT GTTTTACAT ACTT 3' (complementary to nt. nos 691 to 714)
HPUIS¹ 5' GAGTTGATTG ACATTGGCGG TAAC 3' (nt. nos. 553 to 576)
HPU3 5' TTTGAAGTGA ATAGATGCTT AGAC 3' (nt. nos. 442 to 465)
30 HPU4 5' GGCTTGCCTA TCAACCAACG CGTT 3' (complementary to nt. nos 595 to 618)
HPU5 5' GGCCGGTTCA TCGCATTGAG TCAA 3' (nt. nos 2074 to 2097)
HPU6 5' CTTTATTGGC TGGTTAGAG TTAC 3' (complementary to nt. nos 2374 to 2397)
35 HPU1L 5' GGGCTTGAAA GACAAGTGT GCCG 3' (nt. nos 2242 to 2265)
HPU7 5' TACAGAGAAA TGTTCGCTCA TCAT 3' (nt. nos 2143 to 2166)

- 8 -

HPU8 5' GACTTCAATG TGAGCGGTAG TGTC 3' (complementary to nt. nos 2311
to 2334)

HPU9 5' ACTTTCGGTA AACGCTTAGA CATT 3' (nt. nos 481 to 504)

HPU10 5' CTCTTTAGCT CTGTGTAAAG CAAT 3' (complementary to nt. nos 637
5 to 660)

HPU11 5' TTTTACCGGC AACACTTGTC TTTC 3' (complementary to nt. nos 2248
to 2271)

HPU13 5' AATGCCTTTG TCATAAGCCG CTTG 3' (complementary to nt. nos 2206
to 2229)

10 (Footnote: HPU1¹, HPU2¹ and HPUIS¹ are the same sequences as HPU1, HPU2
and HPUIS above, but increased in length by six nucleotides to a total
of 24).

23 units

15 (037) 5' GCACCAAGCTT CAATTTGATC GGC 3'

18 Units

(HPU54) 5' TGGGATTAGC GAGTATGT 3' (nt. nos. 1971 - 1988)

(DY3) 5' GCAAGCATGA TCCATGAA 3'

20 (DY4) 5' AACGAAAGCA AAAAATT 3'

(DY5) 5' CATGGCGCTA AAAGCGAT 3'

(DY6) 5' AGAGCGGCTG AAGAATAT 3'

(DY7) 5' GGCATTAAAG AAGAATTA 3'

(DY8) 5' CTAAACCAGC CAATAAAG 3'

25 (DY9) 5' GTCAACGGAT CTCGTTAT 3'

(DY10) 5' ATCTCTTCAA GGAAAAAC 3'

(DY11) 5' ACTTTAAGAA TAGGAGAA 3'

(DY12) 5' GCTTGGCGCA ACTCTTTA 3'

(DY13) 5' GCAACGCTTC CTTAAATC 3'

30 (DY14) 5' GTCAATTAC TATTTTC 3'

(DY15) 5' ATGATTAGCT CAAGAAC 3'

(DY16) 5' AAGGTGCGTT TGTTGTAA 3'

(DY17) 5' GGCAATGCTA GGACTTGT 3'

(DY18) 5' ATCAGCAATG GGATTGTC 3'

- 9 -

17 Units

5 (016) 5' GATGTGATGG ATGGCGT 3'
 (017) 5' ACTTTATTGG CTGGTTT 3'
 (025) 5' TGGTTTGAGG GCGAATC 3'
 (030) 5' CCAGCGATT TGCCATC 3'
 (039) 5' GGGTTTACCC GCGCGCG 3'
 (040) 5' TGAATAGATG CTTAGAC 3'
 (042) 5' AGGTAGAAGA AATTIAA 3'
 (043) 5' CACATGGACA TGCTTAT 3'
10 (050) 5' GAACATGACT ACACCAT 3'
 (051) 5' GCCTTAGATG TTGCAGGA 3'
 (052) 5' GTATTGACAC ACACATC 3'
 (055) 5' AATTGCAGAA ATATCAC 3'

15 16 Units

(053) 5' GTTCGCTGAT TCAAGG 3'
HPUT18 5' CCCATTTGAC TCAATG - 3' (complementary to nt. nos 2102 to
2087)

20

15 Units

HPUT1 5' AGGAGAACGAG ATGA 3'
HPUT2 5' ACTTTATTGGC TGGT 3'

25 Such oligonucleotide sequences are readily assembled using known
oligonucleotide synthesis techniques.

From the above list, certain pairs have been shown to have
particular utilities. For example:

30

i) Primer pair 040 + 039 has been shown to be useful on fresh
gastric biopsies and amplify small fragments of 110bp and 127 bp
respectively.

ii) Primer pairs 018 + 054 and 017 + 055 have been shown to be
useful on fresh and stored paraffin embedded gastric biopsies.

35 iii) The following particular H. pylori DNA sequences are
amplified by the following pairs:

- 10 -

Amplified DNA fragment

	Length bp	Nucleotide Nos.	PCR primer pairs
5	411 ¹	304 - 714	HPU1 and HPU2
	162	553 - 714	HPU1S and HPU2
	177 ¹	442 - 618	HPU3 and HPU4
	324 ²	2074 - 2397	HPU5 and HPU6
	156	2242 - 2397	HPU1L and HPU6
	192 ²	2143 - 2334	HPU7 and HPU8
10.	234 ³	481 - 714	HPU9 and HPU2
	108 ³	553 - 660	HPU1S and HPU10
	198 ⁴	2074 - 2271	HPU5 and HPU11
	87 ⁴	2143 - 2229	HPU7 and HPU13

A particularly sensitive method of H. pylori detection according to the invention by PCR amplification of genomic H. pylori DNA involves using two pairs of primers to amplify overlapping sequences of H. pylori DNA, the first pair of primers amplifying a first sequence of a given length, and the second pair of primers amplifying a shorter sequence within the overall length of the first sequence. In the above table certain pairings are identified by the pairs of superscripts (1,1), (2,2), (3,3) and (4,4) and these represent overlapping pairs of amplified sequences with the shorter sequence nesting within the longer sequence.

Thus the 177bp sequence amplified by primers HPU3 and HPU4 nests within (i.e. is identical to an intermediate length of) the longer 417bp sequence amplified by primers HPU1 AND HPU2. Similarly the 192bp product of HPU7 and HPU8 nests within the longer 324 bp product of HPU5 and HPU6; the 108bp product of HPU10 and HPU1S nests within the 234bp product of HPU9 and HPU2; and the 87bp product of HPU7 and HPU13 nests within the 198bp product of HPU11 and HPU5.

Although PCR amplification appears to be the presently preferred method of H. pylori detection using the H. pylori specific oligonucleotides of this invention, other detection procedures are available and are well known in the art. To this end the H. pylori

- 11 -

specific oligonucleotides of this invention may be provided with a variety of different labels: radioactive, fluorescent, enzyme, all permitting the detection of any hybridised oligonucleotide bound to the unidentified DNA sample under investigation. Alternatively, of course, 5 for sandwich hybridisation techniques the H. pylori specific oligonucleotides of this invention may be immobilized in any known appropriate fashion, e.g. by binding to a variety of different solid substrates, both particulate, e.g. glass, Sephadex, Sephacryl beads etc., and continuous surface substrates, either chemically or by polyDT 10 or polyDA tailing of the oligonucleotide permitting immobilisation of the oligonucleotide on a polyDA or polyDT coated surface. Such techniques of DNA labelling and immobilization are well known in the art, as are methods for the detection of microorganisms, in general, utilising DNA probes and which will be equally suitable in accordance 15 with the present invention for the specific detection of H. pylori. Such procedures and methods are not part of the present invention as such and need not be further described here, save that, as already indicated, the chain length of from 15 to 50 nucleotides does not include non-complementary sequences added to the oligonucleotide for a 20 specific purpose, e.g. labelling or poly DA or poly DT tailing.

The H. pylori specific oligonucleotides covered by this invention include both single- and double-stranded versions, it being understood that in any subsequent hybridisation procedures such as the detection 25 of H. pylori in the gastric mucosa or other secretions or products of the gastrointestinal tract, such double-stranded probes will require denaturing to provide the probes in single-stranded form.

Also included within the scope of this invention are a method of 30 detecting the presence of H. pylori in a sample especially a saliva sample which comprises treating the sample to release the DNA or mRNA from any H. pylori present in the sample, and probing that released DNA or mRNA for the presence of the H. pylori urease gene using H. pylori specific oligonucleotides according to the invention, and diagnostic 35 kits for the diagnosis of H. pylori infection in a patient comprising an H. pylori specific oligonucleotide according to the invention.

- 12 -

The invention is illustrated by the following examples.

Example 1

5 Construction of Radiolabelled H. pylori specific Oligonucleotide (037)

037 was synthesized on an Applied Biosystems oligonucleotide synthesizer and radiolabelled at its 5' end with T4 polynucleotide kinase and $\Gamma^{32}P$ and then purified.

10

Radioactively labelled probe numbers 016 to 054 were constructed in identical fashion.

Example 2

15

Detection and amplification of H. pylori DNA using oligonucleotide primers 040 and 039.

20 Oligonucleotide primers e.g. 040 and 039, were used at a final concentration of 1 μ M to amplify H. pylori DNA obtained by boiling H. pylori cells in 50 μ l of H_2O for 10 minutes. The DNA was amplified using a cycle profile of 94°C 1 min., 31°C 1 min., and 72°C 3 min. After the last cycle the polymerisation step was extended from 3 to 10 minutes. Twenty six cycle of amplification were performed in total.

25 The reactions were carried out in 100 μ l volumes, 20 μ l samples were run on agarose gels and amplified DNA detected by ethidium bromide staining and comparison with mol. wt. standards. At least 1 ng of DNA was required to give an amplified fragment. By this method H. pylori DNA has been amplified from cell populations as low as 10 H. pylori cells, 30 thus indicating the extreme sensitivity of H. pylori DNA probes according to this invention. Simultaneous controls with C. jejuni and C. coli have failed to produce any evidence of amplification, indicating the specificity of probes according to the present invention of H. pylori.

35

Confirmation that the amplified fragments are H. pylori DNA is obtained both by hybridisation with internal oligonucleotide probes and

- 13 -

by using two internal primers for PCR after initial amplification, e.g. by using probes 016 and 037, to give amplified fragments of 1.1 kb, and then using 5% of this reaction product to perform further PCR with probes 040 and 039 to give a 0.4 kb fragment.

5

Example 3.

Example 2 was repeated using the two primers (HPU1) 5'-GCCATGGTA
10 AATTAGTT-3' and (HPU2) 5'-CTCCTTAATT GTTTAC-3' amplifying the 411 bp
fragment of H. pylori urease gene (nucleotide sequence nos: 304-714) on
various dilutions of H. pylori, the urease clone DNA pTCP3, and 6 human
gastric biopsy samples, but with a slightly modified PCR cycle, namely:
95°C 5 min., 94°C 1 min., 50°C 1 min., and 72°C 3 min. The accompanying
15 photograph (Figure 1) shows the resulting ethidium-bromide stained
agarose gel. Lane 1 represents the urease clone DNA pTCP3; lanes 2-8
10-fold dilutions of H. pylori (630) from 10⁶ bacteria (lane 2) down to
zero (lane 8) and lanes 9-14 the six gastric biopsy samples. A 1kb.
ladder was used as standard. Lanes 2-7 show that H. pylori populations
20 as low as 10 bacteria can be detected by the method of the invention
using these primers. Lanes 9-14 show a positive diagnosis in two out
of the four biopsy samples, lanes 9 and 13.

A similar 411bp DNA fragment has been shown to be amplified from
25 a total of 40 different H. pylori strains. H. mustelae and other
urease positive bacteria are PCR negative under similar conditions.

As confirmation, the separated 411 bp bands were transferred by
blotting and probed with a ³²P labelled oligonucleotide probe HPUIS (nt.
30 nos 559 to 576) which is internal to the 411 bp fragment amplified by
HPU1 and HPU2. after washing to remove unbound probe the bound probe
was visualised by exposure to a photographic plate. The resulting
autoradiograph is shown in Figure 2 of the accompanying drawings, and
confirm the identity of the 411bp product.

35

- 14 -

In a separate series of experiments PCR products were amplified using the same PCR protocol and the same pair of primers HPU1 and HPU2, and also the pair HPU18 and HPU54, over a range of other bacteria including H. pylori 2022 (Lane 3), H. Mustelae W0831 (Lane 4),
5 H. Mustellae F6 (Lane 5), Proteus Mirabilis 5 (Lane 6), Morganella Morganii (Lane 7), Providencia retgeri C (Lane 8), Klebsiella pneumoniae (Lane 9), Yersinia entercolitica B2 (Lane 10), Urease positive Campylobacter 88/12830 (Lane 11), Campylobacter jejuni pLA466 (Lane 12), Campylobacter coli pIP1433 (Lane 13) and Wolinella succinogenes 11488 (Lane 14).

The results are shown in the photograph presented as Figure 3 where A shows the results obtained with HPU1 and HPU2, and B shows the results obtained with HPU54 and HPU18. The 411 bp fragment
15 characteristics of H. pylori is clearly to be seen in Lane 3 of A in marked contrast to the other Lanes, likewise the 110 bp fragment amplified by HPU54 and HPU18 (Lane 3 of B). Lane 1 represents a 1kb mol. wt. standard, and Lane 2 represents a negative control lane.

20 Using the same pair of primers (HPU54 and HPU18) and the same PCR protocol, PCR products were amplified from paraffin stored gastric biopsy sections (section nos. 4593, 10412, 7213, 4591 and 1841 - Lanes 3 - 7 Figure 4). Lane 2 represents a positive control paraffin embedded H. pylori 630, Lane 9 a negative control and Lane 8 a 1kb mol.
25 wt. standard ladder. Lane 1 shows a PCR product of 100 bp amplified from section 4593 by human β -globulin primers. Lanes 3 - 6 clearly show the 110 bp fragment amplified by the primers HPU18 and HPU54 confirming the presence of H. pylori in sections 4594 10412 7213 4591 (Lanes 3 - 6) and its absence from section 1841 (Lane 7).

30

35

- 15 -

APPENDIX

5 SEQ. ID. NO. 1

Sequence Type: Nucleotide with corresponding amino acid
Sequence Length: 2767 bp
10 Strandedness: Double
Topology: Linear
Molecule Type: Genomic DNA
Original Source
Organism: H. pylori
15 Immediate
Experimental Source:
Recombinant dEMBL 3 clone dCP2
and sub-cloning into E. coli vector pUC18

20

Features:	-63 to -57	upstream vector linking sequence
	-56 to -1	non-coding upstream region including ribosome binding site etc.
25	1 to 714	sub-unit A protein 27.657 KDA
	715 to 717	stop codon
	718 to 720	non-coding codon
	721 to 2397	Subunit B protein 60.473KDA
	2398 to 2400	stop codon
30	2400 to 2690	non-coding downstream region
	2691 to 2704	downstream vector linking sequences

35

- 16 -

-60 -50 -40 -30 -20 -10

CCT GCAGGTCAAC GGATCTCGTT ATGTCTTCAA GGAAAAACAC TTAAGAATA GGAGAAATGAG
m13

15	30	45	60
ATG AAA CTC ACC CCA AAA GAG TTA GAC AAG TTG ATG CTC CAC TAC GCT GGA GAA TTA GCT	-Met lys leu thr pro lys glu leu asp lys leu met leu his tyr ala gly glu leu ala		
75	90	105	120
AAA AAA CGC AAA GAA AAA GGC ATT AAG CTT AAC TAT GTG GAA GCG GTA CGT TTG ATT AGT	lys lys arg lys glu lys gly ile lys leu asn tyr val glu ala val arg leu ile ser		
135	150	165	180
GCC CAT ATT ATG GAA GAA GCG AGA CGT GGT AAA AAG ACT GCG GCT GAA TTG ATG CAA GAA	ala his ile met glu glu ala arg arg gly lys lys thr ala ala glu leu met gln glu		
195	210	225	240
GGG CGC ACT CTT TTA AAA CCG GAT GAT GTG ATG GAT GGC GTG GCA AGC ATG ATC CAT GAA	gly arg thr leu leu lys pro asp asp val met asp gly val ala ser met ile his glu		
255	270	285	300
GTG GGT ATT GAA GCG ATG TTT CCT GAT GGG ACC AAA CTC GTA ACC GTG CAT ACC CCT ATT	val gly ile glu ala met phe pro asp gly thr lys leu val thr val his thr pro ile		
315	330	345	360
GAG GCC AAT GGT AAA TTA GTT CCT GGT GAG TTG TTC TTA AAA AAT GAA GAC ATC ACT ATC	glu ala asn gly lys leu val pro gly glu leu phe leu lys asn glu asp ile thr ile		
375	390	405	420
AAC GAA GGC AAA AAA GCC GTT AGC GTG AAA GTT CCC CCT GTT GGC GAC AGA CCG GTT CAA	asn glu gly lys ala val ser val lys val pro pro val gly asp arg pro val gln		
435	450	465	480
ATC GGC TCA CAC TTC CAT TTC TTT GAA GTG AAT AGA TGC TTA GAC TTT GAC AGA GAA AAA	ile gly ser his phe his phe phe glu val asn arg cys leu asp phe asp arg glu lys		
495	510	525	540
ACT TTC GGT AAA CGC TTA GAC ATT GCG AGC GGG ACA GCG GTA AGG TTT GAG CCT GGC GAA	thr phe gly lys arg leu asp ile ala ser gly thr ala val arg phe glu pro gly glu		

- 17 -

555 570 585 600
 GAA AAA TCC GTA GAG TTG ATT GAC ATT GGC GGT AAC AGA AGA ATC TTT GGA TTT AAC GCG
 glu lys ser val glu leu ile asp ile gly gly asn arg arg ile phe gly phe asn ala
 615 630 645 660
 TTG GTT GAT AGG CAA GCC GAT AAC GAA AGC AAA AAA ATT GCT TTA CAC AGA GCT AAA GAG
 leu val asp arg gln ala asp asn glu ser lys ile ala leu his arg ala lys glu
 675 690 705
 CGT GGT TTT CAT GGC GCT AAA AGC GAT GAC AAC TAT GTA AAA ACA ATT AAG GAG TAA GAA
 arg gly phe his gly ala lys ser asp asp asn tyr val lys thr ile lys glu OCH
 735 750 765 780
 ATG AAA AAG ATT AGC AGA AAA GAA TAT GCT TCT ATG TAT GGC CCT ACT ACA GGC GAT AAA
 met lys lys ile ser arg lys glu tyr ala ser met tyr gly pro thr thr gly asp lys
 795 810 825 840
 GTG AGA TTG GGC GAT ACA GAC TTG ATC GCT GAA GTA GAA CAT GAC TAC ACC ATT TAT GGT
 val arg leu gly asp thr asp leu ile ala glu val glu his asp tyr thr ile tyr gly
 855 870 885 900
 GAA GAG CTT AAA TTC GGC GGC GGT AAA ACC CTA AGA GAA GGC ATG AGC CAA TCT AAC AAC
 glu glu leu lys phe gly gly lys thr leu arg glu gly met ser gln ser asn asn
 915 930 945 960
 CCT AGC AAA GAA GAA CTG GAT CTA ATC ATC ACT AAC GCT TTA ATC GTG GAT TAC ACC GGT
 pro ser lys glu glu leu asp ile ile thr asn ala leu ile val asp tyr thr gly
 975 990 1005 1020
 ATT TAT AAA GCG GAT ATT GGT ATT AAA GAT GGC AAA ATC GCT GGC ATT GGT AAA GGC GGT
 ile tyr lys ala asp ile gly ile lys asp gly lys ile ala gly ile gly lys gly gly
 1035 1050 1065 1080
 AAC AAA GAC ACG CAA GAT GGC GTC AAA AAC AAT CTT AGC GTG GGT CCT GCT ACT GAA GGC
 asn lys asp thr gln asp gly val lys asn asn leu ser val gly pro ala thr glu ala
 1095 1110 1125 1140
 TTA CCC GGT GAA GGT TTG ATT GTA ACT GCT GGT ATT GAC ACA CAC ATC CAC TTC ATC
 leu ala gly glu gly leu ile val thr ala gly gly ile asp thr his ile his phe ile
 1155 1170 1185 1200
 TCC CCC CAA CAA ATC CCT ACA GCT TTT GCA AGC GGT GTA ACA ACC ATG ATT GGT GGC GGA
 ser pro gln gln ile pro thr ala phe ala ser gly val thr thr met ile gly gly
 1215 1230 1245 1260
 ACT GGC CCT GCT GAT GGC ACT AAC GCA ACC ACT ATC ACT CCA GGT AGA AGA AAT TTA AAA
 thr gly pro ala asp gly thr asn ala thr thr pro gly arg arg asn leu lys
 1275 1290 1305 1320
 TTC ATG CTC AGA GCG GCT GAA GAA TAT TCT ATG AAC TTT GGT TTC TTG GCT AAA GGT AAC
 phe met leu arg ala ala glu glu tyr ser met asn phe gly phe leu ala lys gly asn
 1335 1350 1365 1380
 GCT TCT AAC GAT GCA AGC TTA GCC GAT CAA ATT GAA GCT GGT GCG ATT GGC CTT AAA ATC
 ala ser asn asp ala ser leu ala asp gln ile glu ala gly ala ile gly leu lys ile
 1395 1410 1425 1440
 CAC GAA GAC TGG GGC ACC ACT CCT TCT GCA ATC AAT CAT GCG TTA GAT GTT GCG GAC AAA
 his glu asp trp gly thr thr pro ser ala ile asn his ala leu asp val ala asp lys
 1455 1470 1485 1500
 TAC GAT GTG CAA GTC GCT ATC CAC ACA GAC ACT TTG AAT GAA GGC GCG GGT TGC GTG GAA GAC
 tyr asp val gln val ala ile his thr asp thr leu asn glu ala gly cys val glu asp
 1515 1530 1545 1560
 ACT ATG GCA GCT ATT GCC GGA CGC ACT ATG CAC ACT TAC CAC ACT GAA GGC GCT GGC GGC
 thr met ala ala ile ala gly arg thr met his thr tyr his thr glu gly ala gly gly
 1575 1590 1605 1620
 GGA CAC GCT CCT GAT ATT ATT AAA GTG GCC GGT GAA CAC AAC ATC CTA CCC GCT TCC ACT
 gly his ala pro asp ile ile lys val ala gly glu his asn ile leu pro ala ser thr

- 18 -

1635	1650	1665	1680
AAC CCC ACT ATC CCT TTC ACC GTG AAT ACA GAA GCC GAA CAC ATG GAC ATG CTT ATG GTG	asn pro thr ile pro phe thr val asn thr glu ala glu his met asp met leu met val		
1695	1710	1725	1740
TGC CAC CAC TTG GAT AAA AGC ATT AAA GAA GAT GTC CAG TTC GCT GAT TCA AGG ATT CGC	cys his his leu asp lys ser ile lys glu asp val gln phe ala asp ser arg ile arg		
1755	1770	1785	1800
CCT CAA ACC ATT GCG GCT GAA GAC ACT TTG CAT GAC ATG GGG ATT TTC TCA ATC ACT AGT	pro gln thr ile ala ala glu asp thr leu his asp met gly ile phe ser ile thr ser		
1815	1830	1845	1860
TCT GAC TCT CAA GCG ATG GGC CGT GTG GGT GAA GTT ATC ACT AGA ACT TGG CAA ACA GCT	ser asp ser gln ala met gly arg val gly glu val ile thr arg thr trp gln thr ala		
1875	1890	1905	1920
GAC AAA AAT AAA GAA TTT GGC CGC TTG AAA GAA GAA AAA GGC GAT AAC GAC AAC TTC	asp lys asn lys glu phe gly arg leu lys glu glu lys gly asp asn asp asn phe		
1935	1950	1965	1980
AGG ATC AAA CGC TAC TTG TCT AAA TAC ACC ATT AAC CCA GCG ATC GCT CAT GGG ATT AGC	arg ile lys arg tyr leu ser lys tyr thr ile asn pro ala ile ala his gly ile ser		
1995	2010	2025	2040
GAG TAT GTC GGT TCT GTA GAA GTG GGC AAA GTG GCT GAC TTG GTA TTG TGG AGT CCC GCA	glu tyr val gly ser val glu val gly lys val ala asp leu val leu trp ser pro ala		
2055	2070	2085	2100
TTC TTT GGT GTG AAA CCC AAC ATG ATC ATC AAA GGC GGG TTC ATC GCA TTG AGT CAA ATG	phe phe gly val lys pro asn met ile ile lys gly gly phe ile ala leu ser gln met		
2115	2130	2145	2160
GGT GAT GCG AAC GCT TCT ATC CCT ACC CCA CAA CCA GTT TAT TAC AGA GAA ATG TTC GCT	gly asp ala asn ala ser ile pro thr pro gln pro val tyr arg glu met phe ala		
2175	2190	2205	2220
CAT CAT GGT AAA GCT AAA TAC GAT GCA AAC ATC ACT TTT GTG TCT CAA GCG GCT TAT GAC	his his gly lys ala lys tyr asp ala asn ile thr phe val ser gln ala ala tyr asp		
2235	2250	2265	2280
AAA GGC ATT AAA GAA GAA TTA GGG CTT GAA AGA CAA GTG TTG CCG GTA AAA AAT TGC AGA	lys gly ile lys glu glu leu gly leu glu arg gln val leu pro val lys asn cys arg		
2295	2310	2325	2340
AAT ATC ACT AAA AAA GAC ATG CAA TTC AAC GAC ACT ACC GCT CAC ATT GAA GTC AAT TCT	asn ile thr lys asp met gln phe asn asp thr thr ala his ile glu val asn ser		
2355	2370	2385	2400
GAA ACT TAC CAT GTG TTC GTG GAT GGC AAA GAA GTA ACT CTA AAC CAG CCA ATA AAG TGA	glu thr tyr his val phe val asp gly lys glu val thr leu asn gln pro ile lys OPA		
2410	2420	2430	2440
GCTGGCGCA ACTCTTTAGC	ATTTCTAGG	ATTTTTAGA	GCAACGCTTC
2480	2490	2500	2510
CTCTCTGATT TTTTGTATT	CAAAAAATTG	GGGGCTTTT	TTGTTTTAT
2550	2560	2570	2580
TTCTTTATGA TTAGCTCAAG	CAACAAAAGT	TATTCTGAAG	GTGCGTTGT
2620	2630	2640	2650
AGGAAAAGGC AATGCTAGGA	CTTGTATTGT	TATATGTTGG	GATTGTTTA
2690	2700	2704	
GTAAACAAA	<u>GTCGACTCTA</u>	<u>GAGG</u>	

CLAIMS:

5 1. An oligonucleotide having a chain length of from 15 to 50 nucleotide units and showing substantially specific binding affinity to the H. pylori gene encoding the A and B sub-units of H. pylori urease, characterised in that the oligonucleotide comprises a sequence of at least 15 nucleotides which is the same as or complementary to a
10 sequence to be found in a highly conserved region of the gene.

2. An oligonucleotide according to claim 1 having a sequence of at least 15 nucleotides which is the same as or complementary to a sequence of at least 15 nucleotides to be found in the regions of the
15 gene running from nt. nos 255 - 714, 720 - 1020 or 1950 - 2397 inclusive.

3. An oligonucleotide according to claim 1 or 2 having a chain length of from 15 to 25 nucleotides.
20

4. An oligonucleotide according to claim 1, 2 or 3, having specific binding affinity for a sequence of nucleotides at the 5' or 3' ends of an approximately 411 bp fragment of the H. pylori urease gene running from about nt. no. 298 to 714 inclusive, or an approximately 324 bp
25 fragment running from about nt. no. 2074 to nt. no. 2397, or an approximately 110 bp fragment running from about nt. 1971 to about nt. no. 2102.

5. An oligonucleotide according to claim 1, comprising any one of
30 the oligonucleotides hereinbefore identified by number.

6. The oligonucleotides:

35 5'-GCCAATGGTA AATTAGTT-3'
5'-ATTGAGGCCA ATGGTAAATT AGTT-3'
5'-CTCCTTAATT GTTTTAC-3'
5'-CTCCTTAATT GTTTTACAT AGTT-3'

- 20 -

5'-ATTGACATTG GCGGTAAC-3'
5'-GGCGGGTTCA TCGCATTGAG TCAA-3'
5'-CTTTATTGGC TGGTTTAGAG TTAC-3'
5'-TGGGATTAGC GAGTATGT-3'
5'-CCCATTTGAC TCAATG-5

5

7. A DNA probe comprising an oligonucleotide according to any one of claims 1 to 6, labelled with a label permitting detection of the oligonucleotide when hybridised to a complementary sequence of the H. pylori urease gene.

10

8. A DNA probe according to claim 7, wherein the label is radioactive, fluorescent or enzyme label attached to the oligonucleotide sequence.

15

9. A DNA probe comprising an oligonucleotide according to any one of claims 1 to 6 immobilized onto a solid support.

10. A method of detecting the presence of H. pylori in a sample, 20 which comprises treating the sample to release the DNA or mRNA from any H. pylori present in the sample, and detecting that released DNA or mRNA for the presence of the H. pylori urease gene using an oligonucleotide according to any one of claims 1 to 6 or a DNA probe according to any one of claims 7 - 9.

25

11. A method according to claim 10, as applied to the detection of H. pylori in a sample from the gastrointestinal tract of a patient.

12. A method according to claim 11, wherein said sample is a gastric 30 mucosa, saliva or faecal sample.

13. A method according to claim 10, 11 or 12, wherein the detection process involves amplification of the released H. pylori DNA by a polymerase chain reaction (PCR) using a pair of oligonucleotides 35 according to any one of claims 1 - 6 as primers.

14. A method according to claim 13 wherein the PCR is effected using

one or more of the following primer pairs:

- HPU1 and HPU2
- HPU1¹ and HPU2¹
- 5 HPU1S¹ and HPU2
- HPU3 and HPU4
- HPU5 and HPU6
- HPU7 and HPU8
- HPU9 and HPU2
- 10 HPU10 and HPU1S¹
- HPU11 and HPU5
- HPU54 and HPU18
- HPU7 and HPU13
- HPUT1 and HPUT2
- 15 040 and 039
- 018 and 054
- 017 and 055

15. A method according to claim 13 wherein the PCR is effected using
20 the primer pair:

- (i) 5'-GCCAATGGTA AATTAGTT-3'
and
5'-CTCCTTAATT GTTTTAC-3'
- 25 (ii) 5'-ATTGAGGCCA ATGCTAAATT AGTT-3'
and
5'-CTCCTTAATT GTTTTACAT AGTT-3'
- 30 (iii) 5'-GGCGGGTTCA TCGCATTGAG TCAA-3'
and
5'-CTTATTGGC TGCTTAGAG TTAC-3'
or
(iv) 5'-TGGGATTAGC GACTATGT-3'
- 35 and
5'-CCCATTTGAC TCAATG-3'

- 22 -

16. A diagnostic kit for the diagnosis of H. pylori infection in a patient comprising an oligonucleotide according to any one of claims 1
5 to 5 or a DNA probe according to any one of claims 7 - 9.

17. A diagnostic kit according to claim 15, which is a PCR kit comprising a pair of oligonucleotides as primers.

10 18. A diagnostic kit according to claim 17 wherein the oligonucleotide primers comprise one or more of the primer pairs listed in claim 14.

19. A diagnostic kit according to claim 17 containing the primer pair
15

(i) 5'-GCCAATGGTA AATTAGTT-3'

and

5'-CTCCTTAATT GTTTTAC-3'

20 (ii) 5'-ATTGAGGCCA ATGGTAAATT AGTT-3'

and

5'-CTCCTTAATT GTTTTACAT AGTT-3'

(iii) 5'-GGCGGGTTCA TCGCATTGAG TCAA-3'

25 and

5'-CTTTATTGGC TGGTTTAGAG TTAC-3'

or

(iv) 5'-TGGGATTAGC GAGTATGT-3'

and

30 5'-CCCATTTGAC TCAATG-3'

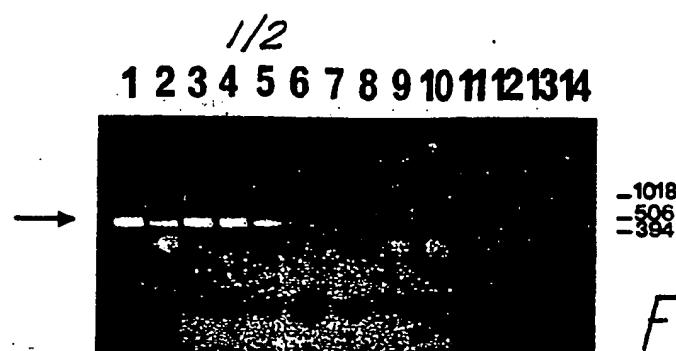


FIG. 1



FIG. 2

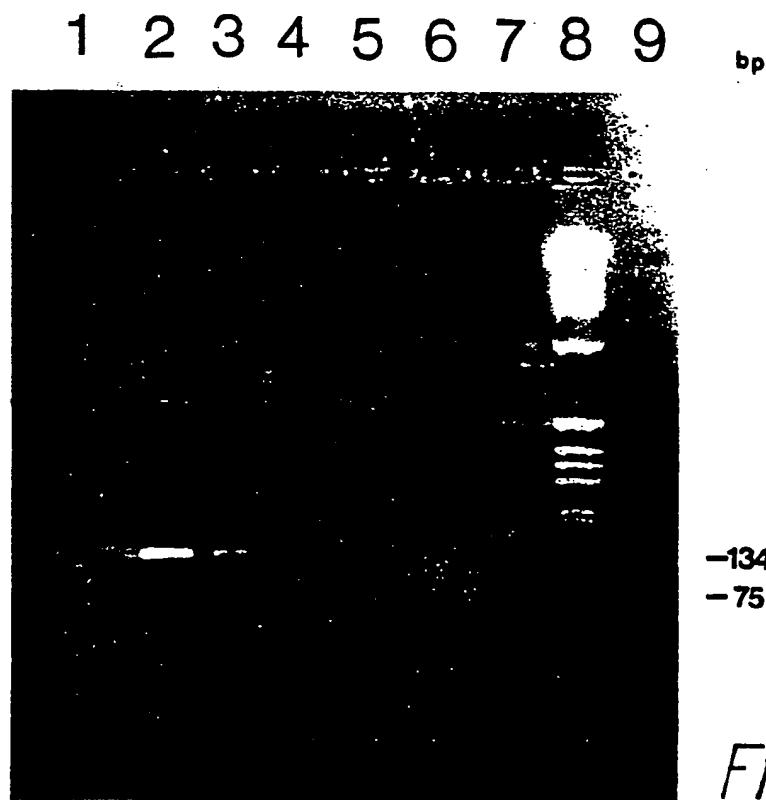


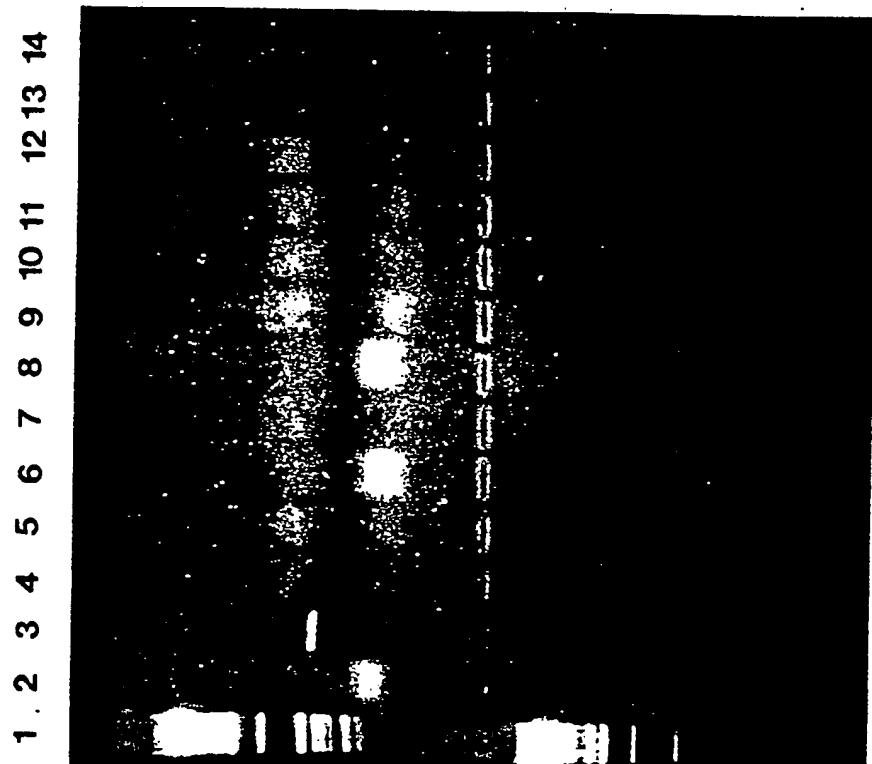
FIG 3

2/2

FIG. 4

A

B

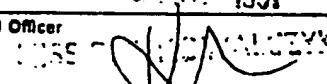


bp

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB 90/01979

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC ⁵ : C 07 H 21/04, C 12 Q 1/68, //C 07 K 15/04		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC ⁵	C 12 Q, G 01 N, C 07 K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT*		
Category ⁹	Citation of Document, ¹⁰ with indication, where appropriate, of the relevant passages ¹¹	Relevant to Claim No. ¹²
X	EP, A, 0367644 (INSTITUT PASTEUR) 9 May 1990 see page 5, lines 6-22 (cited in the application)	1-3, 5, 7-13, 16
A	Infection and Immunity, vol. 57, no. 2, February 1989, American Society for Microbiology, A.L. Clayton et al.: "Molecular cloning and expression of Campylobacter pylori species-specific antigens in Escherichia coli K-12", pages 623-629 see abstract and discussion (cited in the application)	-- 1
A	Chemical Abstracts, vol. 109, no. 7, August 1988, (Columbus, Ohio, US), H.L.T. Mobley et al.: "Characterization of urease from Campylobacter pylori", see page 289, abstract 50580d J. Clin. Microbiol. 1988, 26(5), 831-6	
<p>* Special categories of cited documents: ¹⁰ -----</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the International filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the International filing date but later than the priority date claimed</p> <p>"T" later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
28th March 1991	- 6 April 1991	
International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer 	

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

GB 9001979

SA 42936

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 19/04/91. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A- 0367644	09-05-90	FR-A- WO-A-	2637612 9004030	13-04-90 19-04-90